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Direct and Long-Range Action of a DPP Morphogen Gradient

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Summary

During development of the Drosophila wing, the *decapentaplegic* (*dpp*) gene is expressed in a stripe of cells along the anteroposterior compartment boundary and gives rise to a secreted protein that exerts a long-range organizing influence on both compartments. Using clones of cells that express DPP, or in which DPP receptor activity has been constitutively activated or abolished, we show that DPP acts directly and at long range on responding cells, rather than by proxy through the short-range induction of other signaling molecules. Further, we show that two genes, *optomotor-blind* and *spalt* are transcriptionally activated at different distances from DPP-secreting cells and provide evidence that these genes respond to different threshold concentrations of DPP protein. We propose that DPP acts as a gradient morphogen during wing development.

Introduction

The term morphogen was initially defined by Turing (1952) as a "form generating substance" that is expressed by some cells and moves through surrounding tissue providing other cells with information about their relative position. The concept of a morphogen has been especially valuable in developmental systems that behave as if growth and patterning are controlled by gradients of signaling molecules that emanate from a localized source and trigger distinct responses at different distances (Lawrence, 1966; Stumpf, 1966; reviewed by Lawrence, 1972; Wolpert, 1989). It has been suggested that in such systems the concentration of the putative morphogen declines in a continuous and predictable fashion as it moves away from cells that express it, providing a series of concentration thresholds that control the behavior of surrounding cells as a function of their distance from the source.

Despite the explanatory value of morphogen gradients, considerable controversy remains over whether such gradients actually operate during animal development and, if so, how they organize cell behavior. At least in the early Drosophila embryo, several factors, especially the proteins Bicoid and Hunchback, have attributes expected of classical gradient morphogens (reviewed by St Johnston and Nüsslein-Volhard, 1992). However, the early Drosophila embryo is unusual because it is a syncytium in which cytosolic proteins such as Bicoid and Hunchback, which generally function as DNA-binding transcription factors, can diffuse within a common cytoplasm. Hence, these proteins may not serve as useful precedents for gradient morphogens operating in tissues composed of cells.

During the past 10 to 15 years, several families of secreted proteins have been identified that appear to exert long-range organizing activities on cell proliferation and patterning within tissues. Most prominent among these are members of the Hedgehog (HH), Wnt, and transforming growth factor β (TGFβ) families (reviewed by Ingham, 1994; Siegfried and Perrimon, 1994; Wall and Hogan, 1994). However, the way in which these molecules achieve their long-range organizing influence remains unknown. One possibility is that they function as gradient morphogens, as described above. However, another equally plausible possibility is that they act as short-range inducers that initiate sequential chains, or relay systems, of secondary signals that dictate distinct responses at different positions.

To date, much of the available experimental data seem to argue against the view that these molecules function as morphogens. The long-range organizing activity of Drosophila HH, for example, seems to be propagated indirectly by its ability to act as a short-range inducer of Decapentaplegic (DPP) and Wingless (WG) (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995; Zecca et al., 1995; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995), and the same may also be true for Sonic hedgehog in vertebrate limb development (Johnston and Tabin, 1995). In the case of Wnts and the Drosophila TGFβ-related protein DPP (Padgett et al., 1987), there is compelling evidence that these can serve as local inducers, as shown for WG and DPP in the Drosophila embryo (Ferguson and Anderson, 1992; Vincent and Lawrence, 1994; Bienz, 1994) or for Xwnt-8 in Xenopus axis formation (Smith and Harland, 1991; Parkin et al., 1993). However, in the few apparent cases of longer-range organizing activity (e.g., Struhl and Basler, 1993; Hoppler and Bienz, 1995; Zecca et al., 1995; Gurdon et al., 1994; Fan and Tessier-Lavigne, 1994), the evidence that these molecules normally exert a direct influence on responding cells is not compelling. The failure to obtain such evidence has therefore left uncertain whether any extracellular signaling molecules actually function as bona fide gradient morphogens (e.g., Vincent, 1994).

Here, we have sought to resolve this uncertainty for the case of DPP. The strategy we have chosen is to compare the consequences of ectopically expressing DPP with those of ectopically activating the receptor system that normally transduces DPP. If DPP operates indirectly through the induction of other signals, then the ectopic activity of the receptor system alone should be as effective as ectopic expression of the ligand in exerting a long-range influence on surrounding tissue. By contrast, if DPP operates as a gradient morphogen, only ectopic activity of the ligand, and not that of its receptor system, should have this property.

To apply this experimental test, we have generated conditions that lead to ectopic, constitutive activity of the type I and type II receptors Thick veins (TKV) and Punt, which are both essential for transducing all known responses to DPP in Drosophila (Ruberte et al., 1995). In addition, we have identified two genes, *optomotor-blind* (*omb*) and *spalt*, that appear to respond to different levels of DPP signaling. We show that cells in which the DPP receptor system is constitutively activated express both *omb* and *spalt*, but do not induce ectopic expression of these genes in neighboring cells. In contrast, cells that ectopically express DPP not only transcribe both genes, but also induce their transcription in overlapping but distinct populations of surrounding cells. These and additional findings we report provide a strong argument that DPP acts directly on cells at a distance from DPP-secreting cells and suggest that different threshold concentrations of DPP elicit distinct molecular outputs. Hence, DPP appears to exert a long-range influence on wing development by acting as a gradient morphogen, rather than as a short-range inducer of other signals.

Results

Ligand-Independent, Constitutive Signaling by the DPP Receptors Punt and TKV

Like other members of the TGFβ superfamily, DPP signals through heteromeric receptor complexes formed by two transmembrane serine/threonine kinases termed type I and type II receptors (Massagué et al., 1994). They are encoded by the genes *thick veins* (*tkv*; type I) and *punt* (type II) (Nellen et al., 1994; Brummel et al., 1994; Penton et al., 1994; Ruberte et al., 1995; Letsou et al., 1995). Mutations that abolish the activity of either gene completely block DPP signaling (Ruberte et al., 1995). We have used two general approaches to activate these receptors constitutively. First, it has been observed for receptor tyrosine kinases that mere overexpression can activate the Ras/Raf signal transduction pathway in the absence of ligand (e.g., Di Fiore et al., 1987; Basler et al., 1991). Hence, we overexpressed the wild-type forms of either or both TKV and Punt. Because the activity of such overexpressed wild-type receptors might still be ligand dependent, we also overexpressed chimeric forms of these receptors in which the extracellular domains, which include the DPP-binding sites, as well as the transmembrane domains have been replaced with the corresponding domains of the unrelated transmembrane receptor Torso (TOR; Dickson et al., 1992). The second approach we have taken is to overexpress a TKV mutant receptor that has a single amino acid change (Q253D) in the GS domain of TKV and hence resembles a mutant form of the type I TGFβ receptor that has constitutive activity (Wieser et al., 1995). Both approaches were initially tested using well-defined assays for DPP signaling in embryos as described below, and both resulted in constitutive transducing activity.

During early embryogenesis, *dpp* is normally expressed along the dorsal surface of early embryos, where it appears to specify the formation of dorsal as opposed to ventral ectoderm. In embryos that express an upstream activating sequence–*dpp* (*UAS–dpp*) transgene in most ectodermal cells under the control of the *GAL4* driver gene *69B* (Brand and Perrimon, 1993), ventral cells are dorsalized, as shown in Figure 1A (Staehling-Hampton et al., 1994a). Although we observed no effect on dorsoventral pattern when *UAS–tkv*, *UAS–punt*, *UAS–tor–tkv*, or *UAS–tor–punt* genes were singly expressed under *69B* control (Figure 1B; data not shown), coexpression of either the wild-type or chimeric forms of the type I and II receptors caused a strong dorsalization of the embryo. Similar results were also obtained when a *UAS–tkv*^{Q253D} gene was expressed alone under *69B* control. As shown in Figures 1C and 1D, such embryos do not develop ventral denticle belts, but exhibit dorsal hairs along the entire dorsoventral axis. Later in embryogenesis, *dpp* is expressed in a restricted domain within the visceral mesoderm and controls the localized expression of the homeotic gene *labial* in adjacent cells of the underlying endoderm (Bienz, 1994). Ubiquitous expression of *dpp* (under the indirect control of a heat shock promoter) leads to an expansion of the *labial* domain in the midgut such that a substantial fraction of endodermal cells accumulate high levels of Labial protein (Ruberte et al., 1995; Staehling-Hampton and Hoffmann, 1994). Similarly, as shown in Figures 1G–1J, heat shock–induced expression of TKV^{Q253D} or coexpression of both wild-type or chimeric receptors also caused an expansion of Labial expression (compare Figures 1I and 1J with 1G and 1H). Thus, joint overexpression of both wild-type or both TOR-chimeric receptors can suffice to cause a gain of DPP signal transducing activity, whereas overexpression of a constitutively active form of just the type I receptor is sufficient on its own. These findings support the proposal (Wrana et al., 1994; Wieser et al., 1995) that type I and type II receptors function in a heteromeric complex in which the type I receptor acts downstream of the type II receptor.

The ectopic receptor activity associated with joint overexpression of the TOR-chimeric receptors appears indistinguishable from that caused by overexpressing both wild-type receptors or just the TKV^{Q253D} mutant receptor. Because wild-type and TKV^{Q253D} receptors have extracellular domains capable of binding DPP whereas the chimeric receptors do not, we infer that the activity in each case is ligand independent. To confirm this, we examined the consequences of jointly overexpressing both chimeric receptors in a *dpp* null mutant background. As shown in Figure 1E, *dpp* mutant embryos differentiate bands of ventral denticles, which extend circumferentially around the entire dorsoventral axis. In contrast, when both chimeric receptors are overexpressed in *dpp* mutant embryos, these embryos form circumferential bands of dorsal hairs (Figure 1F). Thus, the receptors TKV and Punt are not only required together to transduce all known DPP signaling events that have been assayed (Ruberte et al., 1995), but their ectopic activity can suffice to elicit DPP responses even in the absence of ligand. These findings therefore provide the means to activate the DPP signal transduction pathway irrespective of the ligand and hence to distinguish between gradient morphogen and local inductive explanations for the long-range effects of DPP during limb development.

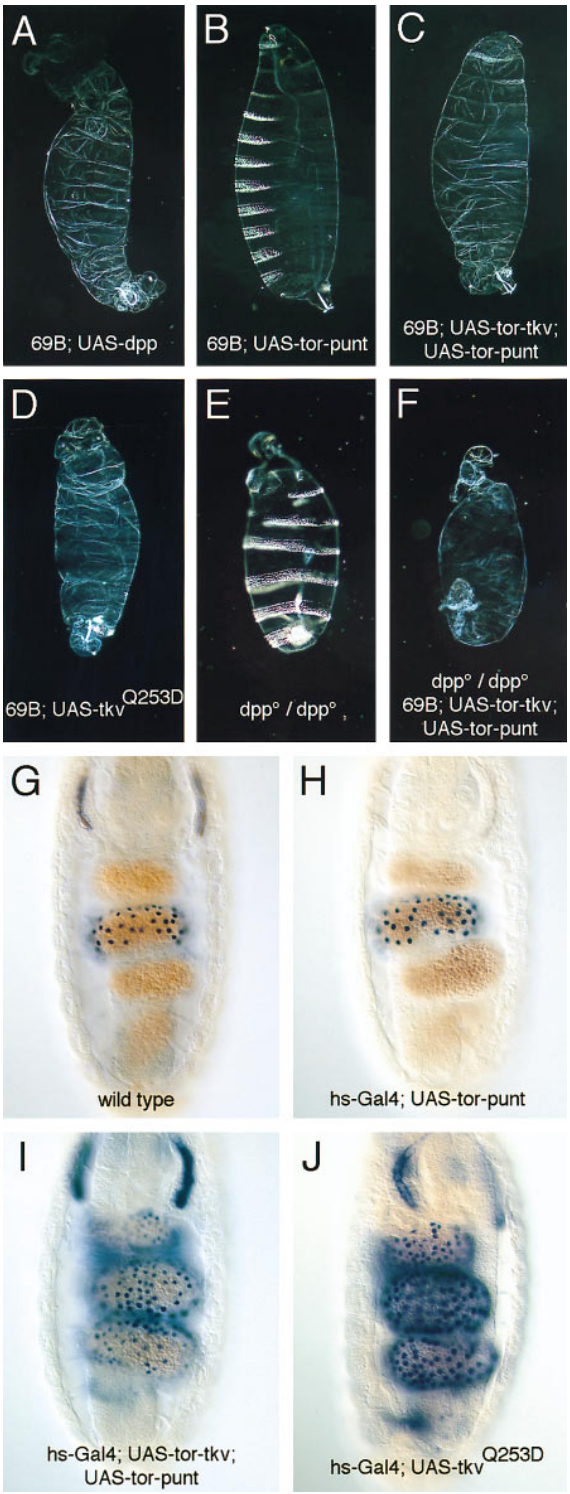

Figure 1. Overexpression of Chimeric or Mutant Forms of the DPP Receptors Punt and TKV Results in Constitutive Activity of the DPP Signal Transduction Pathway

Cuticular phenotypes of embryos overexpressing receptor transgenes in wild-type (A–D) or *dpp* mutant backgrounds (E and F). Anterior is up, and dorsal is to the right.

(A) Ectopic *dpp* expression in most ectoderm cells of developing embryos (under the control of *GAL4* line *69B*) results in dorsalization. Ventral denticle belts are missing; dorsal hairs are found along the entire dorsoventral axis.

(B–D) *69B*-driven expression of the *tor–punt* transgene alone (or *tor–tkv*; data not shown) has no effect on embryonic patterning. Strong dorsalization is observed in embryos coexpressing *tor–tkv* and *tor–punt* (C), coexpressing the wild-type forms of *tkv* and *punt* (data not shown), or expressing the tkv^{Q253D} transgene (D).

(E and F) Even in a *dpp* null mutant background (dpp^{H61}), coexpression of *tor–tkv* and *tor–punt* under the control of *69B* dorsalizes the embryonic ectoderm: bands of dorsal hairs are formed at the expense of the circumferential ventral denticles. The same result was obtained with a *UAS–dpp* transgene (data not shown).

(G–J) Expression of the DPP-responsive gene *labial* (shown in blue) was monitored 4 hr after ubiquitous transgene induction. Coexpression of both wild-type (data not shown) or both *tor*-chimeric receptors (I) or expression of tkv^{Q253D} alone (J) results in Labial expression throughout most of the midgut endoderm, as is the case upon ectopic expression of *dpp* (data not shown; see Ruberte et al., 1995). Expression of either wild-type or chimeric receptor alone had no effect on Labial expression (e.g., in [H]). Dorsal views are shown; anterior is up.

spalt and *omb* Are Target Genes for DPP Signaling in the Developing Wing

In the wing imaginal disc, *dpp* expression is induced by HH in a stripe of anterior cells along the compartment boundary, from where it appears to organize wing pattern along the anteroposterior axis (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995; Zecca et al., 1995). Based on experiments involving gain or loss of *dpp* expression, we previously proposed that DPP secreted by anterior cells along the compartment boundary exerts a graded influence on cells on both sides of the boundary (Basler and Struhl, 1994; Zecca et al., 1995). If this inference is correct, one would predict that target genes that respond to DPP signaling would be expressed in cells of both the anterior and posterior compartments in a broad stripe centered upon the *dpp* expression domain. Two such genes are *omb* (Grimm and Pflugfelder, 1996) and *spalt* (E. Bier, personal communication). Both genes encode proteins with DNA-binding domains (T domain and zinc finger motifs, respectively; Pflugfelder et al., 1992; Kühnlein et al., 1994) and are expressed in broad stripes that overlie the compartment boundary, raising the possibility that they control the transcription of downstream genes in response to DPP signaling. The *spalt* stripe is confined to the wing blade region, while the *omb* stripe extends further along the compartment boundary into most of the rest of the disc (Figures 2B and 2C). Significantly, the *omb* and *spalt* expression domains have different widths: *omb* is expressed in a very broad stripe covering nearly the entire wing blade region, while the *spalt* stripe is narrower, overlying only cells within and close to the *dpp* expression domain (Figures 2A–2D; see also Figure 6A).

To test whether *omb* and *spalt* respond to DPP signaling, we examined their expression in wing discs in which a *UAS–dpp* transgene is transcribed ubiquitously under the control of a *GAL4* driver gene, *C765*. As shown in Figure 2, ubiquitous DPP expression results in large, overproliferating discs that express *omb* in all cells along the anteroposterior axis (Figure 2E) and *spalt* in all cells of the expanded wing blade region (Figure 2G). Identical

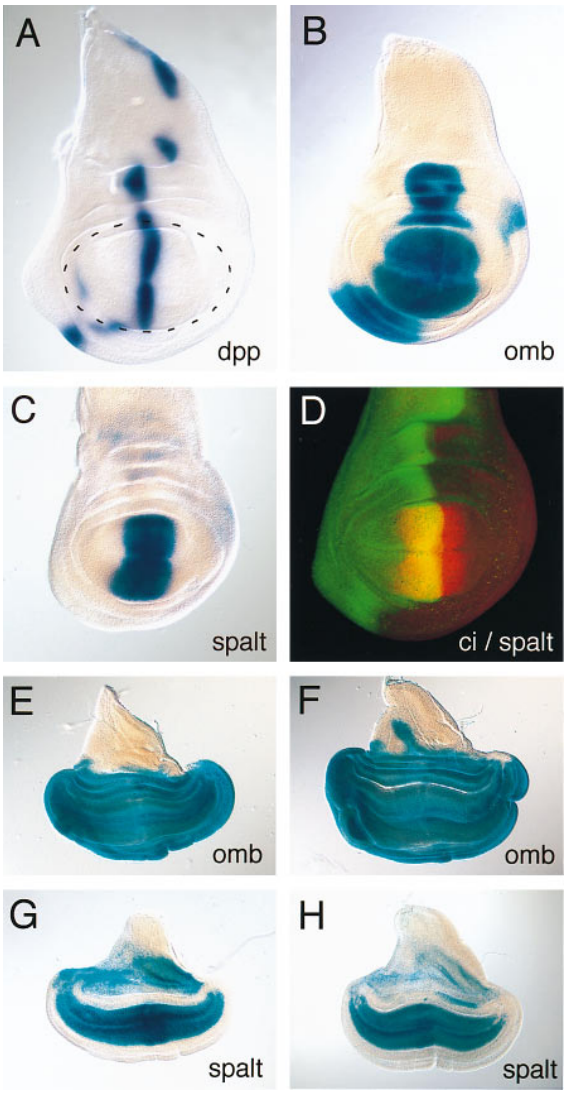

Figure 2. The *omb* and *spalt* Genes Are Targets of DPP Signaling in the Imaginal Wing Disc

(A–C) Wild-type expression pattern of *dpp* (A), *omb* (B), and *spalt* (C), revealed by the *lacZ* reporter genes *BS3.0* (Blackman et al., 1991), *X35* (Sun et al., 1995), and *A405.1M2* (Wagner-Bernholz et al., 1991), respectively. The region that gives rise to the wing blade is indicated by a dashed line in (A). All discs shown in this and subsequent figures are wing discs. Anterior is always to the left, and dorsal is up.

(D) Pattern of *spalt* expression (*spalt–lacZ*; shown in red) in a wild-type disc that is double stained for Cubitus interruptus (CI) protein to mark the anterior compartment (green) and the *dpp* expression domain (higher levels of CI are expressed in the *dpp*-expressing cells along the compartment boundary (Johnson et al., 1995).

(E–H) *omb–lacZ* and *spalt–lacZ* expression in discs that ubiquitously express *dpp* (E and G), both *tor–tkv* and *tor–punt* (F), or tkv^{Q253D} (H) under the control of the *GAL4* enhancer trap gene *C765*. Identical results were obtained following overexpression of tkv^{Q253D} or joint overexpression of *tor–tkv* and *tor–punt*. Such discs are much larger along their anteroposterior axis. They are shown at half the magnification of the discs in (A)–(D). *omb* is expressed in all cells along the anteroposterior axis, except in the most dorsal portion of the disc, where *omb* does not normally appear to respond to DPP signaling (see [B]). *spalt* is expressed in all cells of the prospective wing blade.

results were obtained when the *UAS–dpp* transgene was replaced by the *UAS–*tkv^{Q253D} transgene or by the joint presence of the *UAS–tor–tkv* and *UAS–tor–punt* transgenes (Figures 2F and 2H). Thus, both *spalt* and *omb* appear to be transcribed in response to DPP signaling in the wing. Moreover, as we observed in the embryo, *GAL4*-driven expression of the chimeric and mutant *UAS–tkv* and *UAS–punt* transgenes appears to be at least as potent as that of the *UAS–dpp* transgene in eliciting both responses.

Constitutive Activation of the DPP Receptors Punt and TKV Causes Cell-Autonomous Expression of *spalt* and *omb*

The ability of spatially indiscriminate DPP expression to induce ectopic *omb* and *spalt* expression suggests that during normal development the anteroposterior extent of *omb* and *spalt* expression is determined, directly or indirectly, by DPP. Both *omb* and *spalt* expression could be induced over a long range by the direct exposure of cells to secreted DPP protein emanating from *dpp*-expressing cells along the compartment boundary. Alternatively, movement of DPP from secreting cells might be limited, and exposure to DPP might cause cells to produce one or more secondary signals that spread away from the boundary region and elicit *omb* and *spalt* transcription in cells farther away. As described in the Introduction, we planned to distinguish between these possibilities by assaying the consequences of ectopic activity of the DPP receptor system in defined subpopulations of cells. If *omb* and *spalt* transcription are induced by direct exposure of cells to DPP, such ectopic activity of the receptor system should cause strictly cell-autonomous expression of the two genes. In contrast, if secondary signals are involved, the effect of receptor activation should spread to surrounding wild-type cells, causing them to express *omb* and *spalt* as well. Below, we describe the results obtained when the mutant TKV^{Q253D} receptor is overexpressed in genetically marked clones of wing cells; identical results were obtained when the chimeric forms of both receptors were jointly overexpressed in place of the mutant TKV^{Q253D} receptor.

To obtain marked clones of cells that overexpress the TKV^{Q253D} receptor, we have used a combination of the GAL4/UAS (Brand and Perrimon, 1993) and FLP-out (Struhl and Basler, 1993) techniques for missexpressing genes (see Experimental Procedures). In brief, we generated animals carrying three transgenes: *UAS>CD2,*y^{+}*>*tkv^{Q253D}; the *GAL4* driver *C765*; and *hsp70–flp*. In the *UAS>CD2,*y^{+}*>*tkv^{Q253D} transgene, the *UAS* promoter is separated from the tkv^{Q253D} coding sequence by a FLP-out cassette containing the *CD2* and *yellow*$^{+}$ (y^{+}) marker genes flanked by targets (indicated by angle bracket [>]) for the FLP recombinase. Hence, upon heat shock, a transient pulse of expression of the FLP recombinase can excise the *>CD2,*y^{+}*>* FLP-out cassette, thereby generating clones of *UAS>*tkv^{Q253D} cells that express the tkv^{Q253D} coding sequence under the control of the *GAL4* driver *C765*. Cells within these clones also lack the coding sequence for the reporter protein CD2 and hence can be marked in the disc by the loss of CD2 expression.

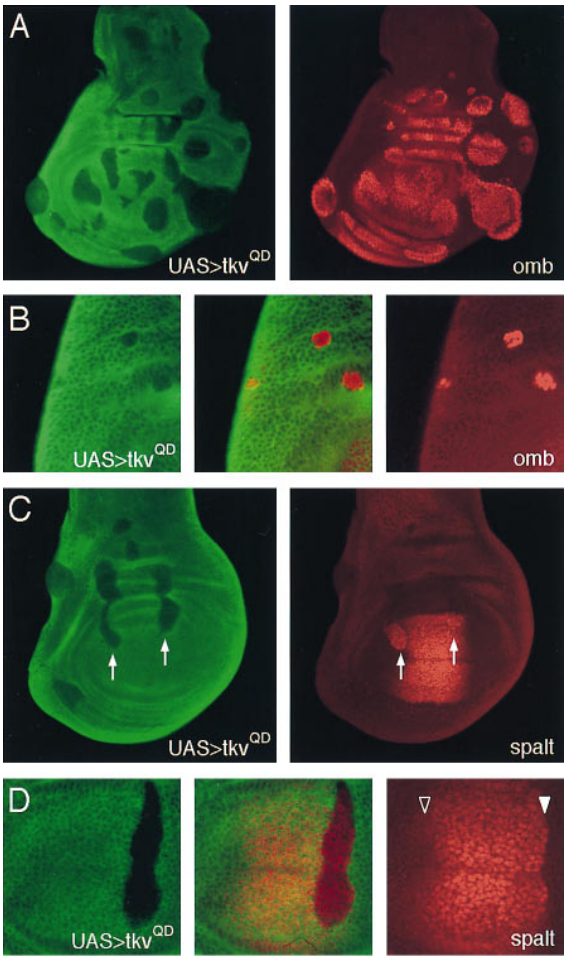

Figure 3. Autonomous Induction of *omb* and *spalt* Transcription in Cells Expressing the Constitutively Active Receptor TKV^{Q253D}

Clones of wing cells overexpressing *tkv*^{Q253D} are shown, marked by the loss of CD2 expression (CD2 is shown in green to the left). *omb* expression is shown in the same discs by double staining for *omb–lacZ* expression (shown in red to the right). Images in (B) and (D) are shown at double the magnification of (A) and (C).

(A and B) In addition to the endogenous *omb* expression in the center of the disc, patches of ectopic *omb* staining are seen associated with the clones. These patches correspond cell by cell to the clones expressing *tkv*^{Q253D}. Clones were induced 72 and 24 hr before staining in (A) and (C), respectively. Clones expressing *tkv*^{Q253D} are consistently larger than clones expressing *dpp* induced at the same stage (see Figure 4). Because large *tkv*^{Q253D} clones tend to bulge out from the epithelium, *omb* staining in such clones can appear nonuniform in the optical sections shown.

(C) In addition to the endogenous pattern, *spalt* is expressed autonomously in *tkv*^{Q253D}-expressing clones (arrows) where these clones comprise wing-blade tissue.

(D) A *tkv*^{Q253D}-expressing clone is shown located at the posterior side (to the right) of the endogenous *spalt* expression domain. Note the graded expression of *spalt* on the anterior side (endogenous *spalt* boundary indicated by open arrowhead) in comparison with the sharp border of *spalt* expression caused by the *tkv*^{Q253D}-expressing clone at the posterior side (closed arrowhead).

As shown in Figure 3A, all cells belonging to *UAS>tkv*^{Q253D} clones express the *omb* gene. Hence, when such clones include cells that fall outside of the normal domain of *omb* expression (which remains normal under these experimental conditions), they invariably exhibit ectopic *omb* expression. However, by double staining for CD2 expression, we observe that ectopic *omb* expression is confined strictly to *UAS>tkv*^{Q253D} cells within each clone: it is not expressed even in immediately adjacent wild-type cells (Figure 3B). This result was consistently observed irrespective of the time of clone induction or the size of the resulting clones. Essentially identical results were obtained for *spalt* (Figures 3C and 3D), the only difference being that *UAS>tkv*^{Q253D} cells only express *spalt* when they arise in the prospective wing blade domain where *spalt* normally responds to DPP (see Figures 2A, 2C, and 2G). Thus, the constitutive activation of the DPP receptor system leads to the autonomous transcription of both *omb* and *spalt* within the same cells, but does not elicit the expression of these genes in surrounding, wild-type cells. Hence, we infer that wing cells that normally express these genes do so because they have received and transduced DPP itself, and not because they have received other signaling molecules induced in response to DPP.

Cells Expressing DPP Organize the Patterns of *spalt* and *omb* Transcription in Surrounding, Nonexpressing Cells

The results of activating the DPP receptor system in marked clones of cells appear to indicate that DPP protein emanating from endogenous *dpp*-expressing cells acts directly on surrounding cells to organize the normal patterns of *omb* and *spalt* expression. To test this inference, we have examined *omb* and *spalt* expression in association with clones that constitutively express DPP. These clones were generated and marked essentially as described above, except that a *UAS>CD2,y*$^{+}$*>dpp* gene was used in place of the *UAS>CD2,y*$^{+}$*>tkv*^{Q253D} gene.

As shown in Figures 4A and 4B, all cells belonging to *UAS>dpp* clones, like those belonging to *UAS>tkv*^{Q253D} clones, invariably express *omb*. However, in striking contrast with *UAS>tkv*^{Q253D} clones, *UAS>dpp* clones also elicit the transcription of *omb* in surrounding wild-type cells, generating broad halos of ectopic *omb* expression when these cells are positioned outside of the normal *omb* expression domain. Similar results were obtained for *spalt* expression (Figure 4C), except that the halos of ectopic *spalt* expression were observed only within the wing blade primordium. Thus, the ability of *UAS>dpp* clones to induce the expression of both genes in surrounding cells can be attributed solely to the direct action of secreted DPP on these cells. It follows from this result that the transcription of the *spalt* and *omb* genes serves as an in vivo assay for DPP signaling. Consequently, several aspects of the patterns of *spalt* and *omb* expression relative to *UAS>dpp*-expressing cells have implications for the organizing activity of DPP.

First, by double labeling for Spalt and *omb* expression, we find that the halos of *omb*-expressing cells surrounding *UAS>dpp* clones are broader than those of Spalt-expressing cells (Figure 4D). The same spatial relationship is observed for the normal boundaries of *spalt* and *omb* expression relative to endogenous *dpp*-expressing cells: *omb* is expressed in a broader stripe

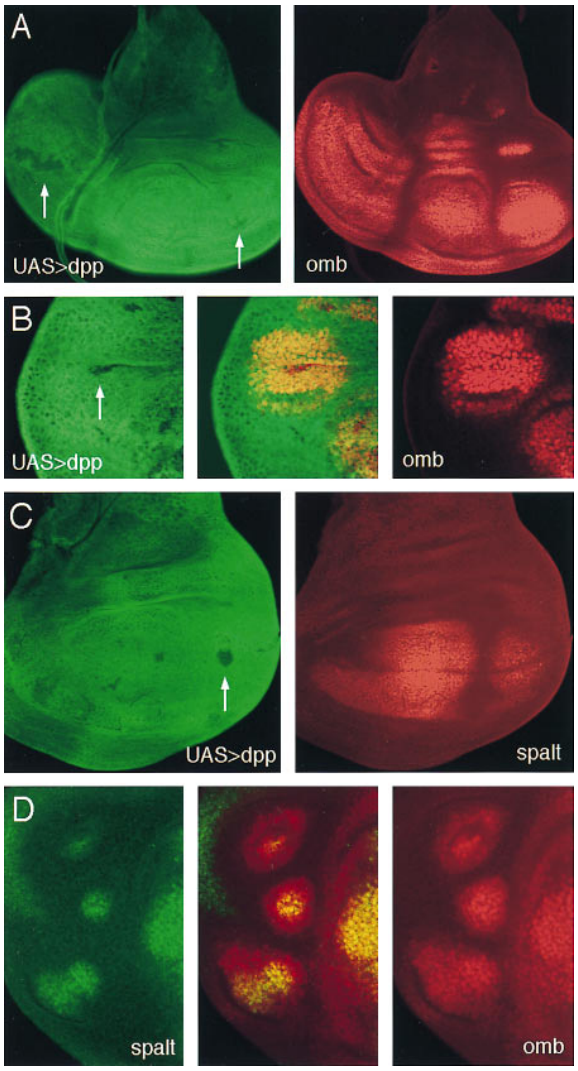

Figure 4. Secreted DPP Acts at Long Range to Induce *omb* and *spalt* Expression in Responding Cells

Clones expressing the *UAS>dpp* transgene are visualized by the loss of CD2 staining (A–C; shown in green to the left). *omb* or *spalt* expression was monitored in the same discs by staining for *omb–lacZ* or *spalt–lacZ* expression (in red to the right). Arrows point to *dpp*-expressing clones. Note that in (A) and (B) the endogenous expression domains of *omb* and *spalt* are visible in addition to the ectopic domains induced by the *UAS>dpp* clones.

(A and B) *dpp*-expressing clones elicit the expression of *omb* within the clones and in surrounding wild-type cells.

(C) *dpp*-expressing clones are associated with *spalt* expression both within and outside of the clone, as long as these cells are located within the wing blade primordium (see Figure 2C).

(D) Part of a wing disc carrying *dpp*-expressing clones. Discs were double labeled for *omb* (*lacZ* expression, shown in red) and Spalt protein expression (in green). In the wing blade region, cells distant from *dpp*-expressing cells of each clone express *omb*, but not Spalt, as is the case for cells on either side of the *dpp* expression domain in wild-type discs (see Figure 6A). Clones were induced 72 hr (A) and between 24 and 48 hr (B–D) before staining. Images in (B) and (D) are shown at double the magnification of those in (A) and (C).

straddling the *dpp*-expressing cells than *spalt* (Figures 2A–2C and 6A). Thus, in general, wing cells appear to respond to DPP in either of two ways, by expressing both genes or only *omb*, depending on how far they are from DPP-secreting cells. A simple explanation that accounts for this distance-dependent response is that cells closer to the *UAS>dpp* cells are exposed to a higher level of secreted DPP protein than cells farther away and hence are instructed to transcribe both genes rather than *omb* alone. Further evidence for such a concentration-dependent mechanism comes from examining the edges of the halos of *spalt* and *omb* expression. In both cases, the edges are not sharp; instead, the level of gene expression declines from peak to undetectable levels over a few cell diameters (Figure 4). Such a graded response contrasts with the sharp boundaries of expression of both genes along the borders of *UAS>tkv*^{Q253D} clones, as illustrated in Figure 3D, and is also observed at the edges of the normal stripes of *spalt* and *omb* expression (Figures 4–6). Hence, it appears that the concentration of secreted DPP protein declines in a graded fashion as a function of distance from *dpp*-expressing cells such that cells along the edges of the *omb* and *spalt* expression domains are exposed to amounts sufficient to induce only intermediate levels of *omb* or *spalt* transcription.

Second, the halos of *omb* and *spalt* expression are generally of constant width around the circumference of the *UAS>dpp* clone, suggesting that all cells around the clone are equally capable of responding to DPP.

Third, the effect of *UAS>dpp* cells on *spalt* and *omb* expression in surrounding cells can extend over a remarkably long distance, up to at least 20 cells in the case of *omb* (e.g., Figure 4A). Similarly, *omb* expression normally extends at least 20 cells both anteriorly and posteriorly beyond the stripe of endogenous *dpp*-expressing cells along the compartment boundary (data not shown). These observations suggest that the range of secreted DPP protein may be quite large, extending through most of the prospective wing blade.

Finally, we observe that late-induced clones of *UAS>dpp* cells, which contain relatively few cells, elicit *omb* expression in surrounding cells only 5–10 cells away (Figure 4B), in contrast with larger clones induced at earlier stages of wing development, which are associated with much broader halos of *omb* expression. This finding suggests that the range of DPP signaling depends on the duration of signaling, the number of cells secreting DPP, or cell proliferation (see Discussion).

TKV Receptor Activity Is Required Autonomously and Continuously for the Ability to Respond to DPP

Although the dramatic difference between the effects of *UAS>dpp* and *UAS>tkv*^{Q253D} cells on surrounding tissue can be viewed as evidence that the patterns of *spalt* and *omb* expression provide a direct visualization of cells that are actively responding to different levels of secreted DPP protein, this need not be the case. An alternative possibility is that the expression of these genes, once triggered by exposure to DPP, may persist even if the responding cells and possibly their descendents are no longer exposed to DPP. To distinguish between these possibilities, we have examined *omb* expression in association with clones of marked cells

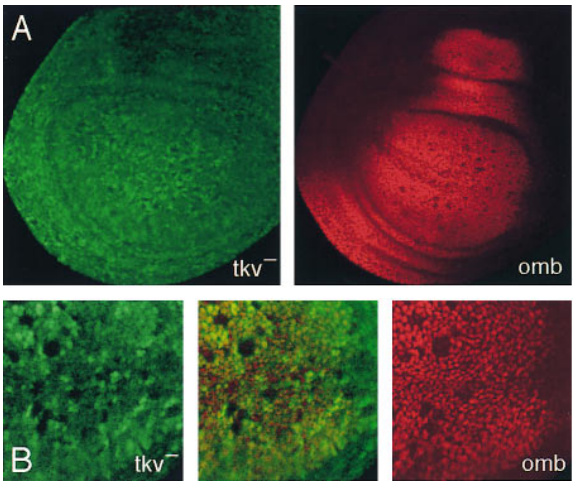

Figure 5. Wing Cells Require *tkv* Gene Function Continuously and Autonomously to Respond to Secreted DPP

(A) A wing disc is shown carrying *tkv* mutant clones induced in mid–third instar larvae. Discs were fixed and double stained 24 hr after clone induction. *omb–lacZ* expression (shown in red at right) is absent in *tkv* mutant cells (marked by the loss of the green πM staining, left).

(B) A 2-fold higher magnification is shown from another disc; the middle frame is a superimposition of the two separate stainings. Note that even cells at the periphery of the normal *omb* domain are still dependent on *tkv* activity for *omb* expression.

that lack endogenous *tkv* gene function (see Experimental Procedures). As shown in Figures 5A and 5B, we find that small clones of mutant cells generated late in disc development but within the normal domain of *omb* expression fail to express *omb*, indicating that DPP input is continuously and autonomously required for *omb* expression. Hence, we infer that *omb*-expressing cells at the edges of the normal domain of *omb* expression both require and continuously receive direct input from DPP.

Concentration-Dependent Control of *omb* and *spalt* Transcription by DPP

Our results so far suggest that DPP protein emanating from secreting cells accumulates as a gradient in surrounding tissue and organizes the patterns of *spalt* and *omb* transcription by triggering the expression of these genes at different concentration thresholds. We have tested this possibility further in two ways.

First, we have asked whether low levels of ectopic DPP expression can activate *omb*, but not *spalt*, transcription. To do this, we have assayed the *omb*- and *spalt*-inducing activity of clones of cells in which the relatively low level constitutive promoter from the *Tubulin* α*1* (*Tub*α*1*) gene is used instead of the *UAS* promoter to drive *dpp* expression (see Experimental Procedures). Larvae carrying the transgenes *Tub*α*1>CD2,y*$^{+}$*>dpp* and *hs–flp* were heat shocked to obtain *Tub*α*1>dpp* clones marked by the absence of the CD2 marker. As shown in Figure 6B, such *Tub*α*1>dpp* clones are frequently associated with *omb* expression; however, they do not appear to express *spalt* (Figure 6C). Hence, the different levels of DPP expression generated under the control of the *Tub*α*1* promoter and the *C765*-driven *UAS* promoter appear to elicit distinct outputs: the former can direct the expression of *omb* without *spalt*, whereas the latter directs the expression of both.

Second, we have asked whether the position of the normal border of *spalt* expression is sensitive to the absolute amount of DPP protein secreted by *dpp*-expressing cells along the compartment boundary. To do this, we have used a transgene in which enhancer sequences from the upstream disc-regulatory region of the *dpp* gene direct GAL4 expression in a manner similar to that of the endogenous *dpp* gene (Masucci et al., 1990; Staehling-Hampton et al., 1994b). In wing discs carrying this *dpp* enhancer–*GAL4* driver gene, we find that the addition of two copies of the *UAS–dpp* transgene significantly extends the domain of *spalt* expression into both the anterior and posterior compartments. As shown in Figures 6D and 6E, this is particularly clear when looking at *spalt* expression in the posterior compartment, because posterior cells do not express either the endogenous *dpp* or *UAS–dpp* gene or the gene *cubitus interruptus* (*ci*), which serves as a marker for the anterior compartment in this experiment. Thus, the boundaries of *spalt* expression appear to depend on the absolute levels of DPP expression generated along the compartment boundary, providing further evidence that the concentration of secreted DPP protein must exceed a second threshold to elicit *spalt* in addition to *omb* transcription in surrounding cells.

Discussion

The controversy between gradient and sequential induction explanations for the control of growth and pattern has a history almost as long as the science of embryology. Initially suggested by Morgan (1897) and Boveri (1902), the gradient concept was subsequently challenged by proponents of inductive mechanisms (e.g., Spemann, 1938). Since then, there have been many well-established examples of short-range inducers, some of which operate in sequential chains. By contrast, there are at present no clear examples of extracellular signaling molecules that have the expected properties of gradient morphogens. The failure to identify such examples continues to undermine the credibility of morphogen gradients as a patterning mechanism.

The results we present here serve to correct this imbalance by providing several lines of evidence that at least one extracellular signaling molecule, DPP, acts as gradient morphogen. Specifically, they establish that DPP secreted by a discrete subpopulation of developing wing cells acts directly and at remarkably long range on surrounding cells and elicits qualitatively distinct outputs from these cells as a function of their distance from the DPP source. The key to demonstrating that DPP works in this way has been the ability to manipulate both the expression of the ligand as well as the activity of the receptor system that transduces it, while assaying the transcription of target genes that normally respond to ligand stimulation. As illustrated by our findings, and by studies that have reached the opposite conclusion for the secreted protein HH (Basler and Struhl, 1994;

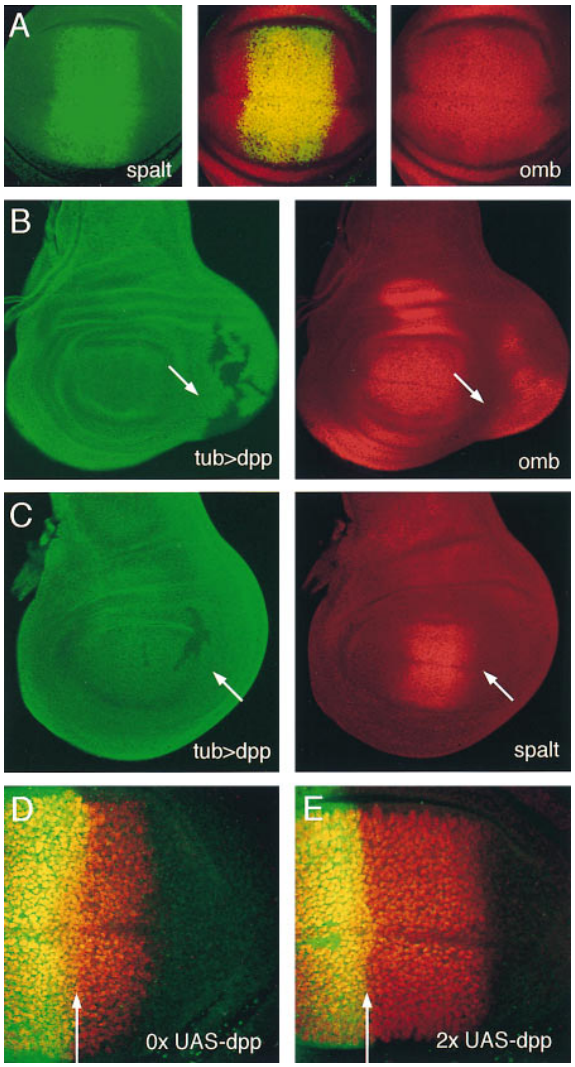

Figure 6. *omb* and *spalt* Expression Are Induced by Different Threshold Concentrations of Secreted DPP

(A) Simultaneous detection of Spalt protein and *omb–lacZ* expression by double staining with antibodies against Spalt protein (green) and *lacZ* (red). Note that the stripe of *omb* expression is broader and straddles that of Spalt.

(B) *Tub*α*1*>*dpp* clones associated with *omb–lacZ* expression. The domain of *omb* expression extends less far from the DPP-expressing cells than in the case of *UAS*>*dpp* clones in a *C765* background (compare with Figure 4A).

(C) A *Tub*α*1*>*dpp* clone is shown that comprises wing blade tissue. In this and all other clones analyzed, we failed to detect ectopic *spalt–lacZ* expression.

(D and E) The normal borders of *spalt* expression depend on the level of *dpp* expressed in anterior cells along the compartment boundary. A *GAL4* line (*blk–GAL4 40C.6*) that expresses GAL4 under the control of a *dpp* imaginal disc enhancer (Masucci et al., 1990; Staehling-Hampton et al., 1994b) was used to drive expression of *UAS–dpp* transgenes within the normal *dpp* expression domain. No GAL4 is produced in cells of the posterior compartment. Long arrows indicate the position of the anteroposterior compartment boundary as determined by the expression of CI (green). No *UAS–dpp* transgene is present in the disc shown in (D); two copies of *UAS–dpp* are present in the disc shown in (E). Note that the posterior border of *spalt* expression is shifted further posteriorly in 2× versus 0× *UAS–dpp* discs (*spalt–lacZ* shown in red).

Zecca et al., 1995; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995), this approach makes it possible to distinguish gradient mechanisms from a variety of other mechanisms, particularly those involving sequential induction. Hence, its future application to other signaling molecules may establish additional examples of gradient mechanisms.

Direct Action of DPP at a Distance from DPP-Expressing Cells

As illustrated in Figure 4, even a small cluster of 10–20 DPP-expressing cells can influence the behavior of hundreds of surrounding cells, some positioned over 20 cells away. Because cells in which the DPP receptor system has been activated fail to induce this response in surrounding cells (Figure 3), whereas cells that respond to secreted DPP continuously require the DPP receptor system to do so (Figure 5), we can attribute this long-range organizing activity solely to the direct action of DPP on responding cells. Thus, secreted DPP must translocate either through or across the tissue over a distance of many cell diameters.

Such an extended range of action was not anticipated for DPP for at least two reasons. First, DPP, as well as bone morphogenetic proteins (BMPs) to which it is closely homologous, are poorly diffusible when expressed in tissue culture and tend to stay bound to the surface of expressing cells and surrounding extracellular matrix (Panganiban et al., 1990). Second, in at least two well-characterized situations, patterning of the dorsal embryonic ectoderm (St Johnston and Gelbart, 1987; Ferguson and Anderson, 1992) and of the embryonic endoderm (Bienz, 1994), the realm of action of DPP appears to be tightly localized to the vicinity in which it is expressed. Indeed in the dorsal ectoderm, *dpp* is transcribed at uniformly high levels in this domain (St Johnston and Gelbart, 1987), and its activity is modulated in a graded fashion by the influx of an antagonistic factor, Short gastrulation (SOG), that appears to emanate from adjacent, more ventral tissue (Francois et al., 1994; Holley et al., 1995). Thus, in both respects, DPP appears to resemble other classes of signaling molecules, such as HH and Wnts, that are either known or thought to function as short-range inducers (e.g., Vincent, 1994).

It is therefore of interest that the movement of DPP may also be severely restricted in the developing wing, even though our results show that it acts directly and at long range in this tissue. In particular, we find that the range of DPP action appears to depend on the duration of signaling: late-induced clones of ectopic DPP-secreting cells have a relatively short-range influence on surrounding cells, in contrast with earlier-induced clones which have a much longer-range influence. Because early-induced clones have more time to proliferate than late-induced clones, this difference could reflect a mass action effect in which the range of signaling depends on the amount of signal generated, which in turn depends on the number of DPP-secreting cells. Alternatively, the movement of DPP away from secreting cells may be limited by its tendency to be sequestered by extracellular matrix components or, possibly, by DPP

receptors or DPP-binding proteins on the surfaces of surrounding cells. Such limits on the movement of DPP may be critical to ensure that it does not spread too far or too fast and hence, as argued previously in general terms (Lawrence, 1966), may play a significant role in allowing DPP to accumulate as a stable concentration gradient of appropriate range and slope. Finally, we note that the correlation between the range of DPP signaling and cell proliferation raises the possibility that secreted DPP may spread away from secreting cells at least in part by being carried along the surface of nonsecreting cells as they proliferate. Such a mechanism would link movement of the signal with cell proliferation, a possibility already suggested by studies of growth and regeneration in other experimental systems (Lawrence et al., 1972).

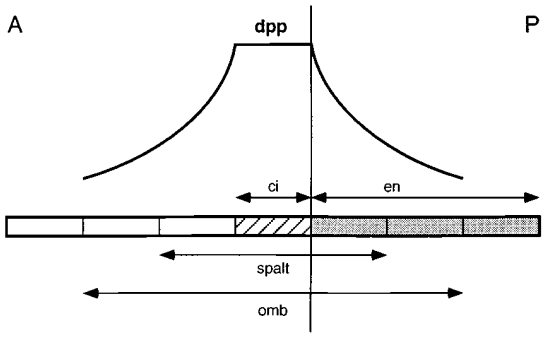

Figure 7. Model for the Organizing Activity of DPP in the Developing Wing

The wing disc (shown as a schematic cross section along the anteroposterior axis) is composed of anterior compartment cells (to the left of the compartment boundary, which is indicated by a vertical line) and *en*-expressing posterior compartment cells (shaded). HH protein produced by posterior cells induces CI protein accumulation and *dpp* transcription in neighboring anterior cells, resulting in a narrow domain of DPP-secreting cells just anterior to the compartment boundary (hatched box; see also Figure 2A). DPP protein emanating from *dpp*-expressing cells accumulates as a concentration gradient and acts directly on responding cells, inducing them to express both *omb* and *spalt* or just *omb*. Hence, the DPP gradient organizes the spatial patterns of *omb* and *spalt* expression by eliciting their transcription at different distances from DPP-secreting cells. The various combinations of *en*, CI, *spalt*, and *omb* expression, all of which encode transcription factors, subdivide the wing primordium into seven distinct zones along the anteroposterior axis.

A Gradient of DPP Elicits Distinct Outputs at Different Threshold Concentrations

Although we have not attempted to visualize the distribution of secreted DPP protein in the developing wing, our results nevertheless provide an indication that the protein accumulates as a concentration gradient and may organize the domains of *spalt* and *omb* expression by inducing their transcription at different concentration thresholds. First, we observe that the edges of the domains of both *omb* and *spalt* induced in response to secreted DPP are not sharp, but rather grade out over a range of a few cell diameters as a function of distance from the secreting cells (Figure 4). Second, we find that the border of *spalt* expression can be shifted further away from DPP-secreting cells by increasing the level of DPP expression in these cells (Figure 6E). Finally, we show that low levels of ectopic DPP expression can induce the transcription of only *omb*, in contrast with higher levels, which induce the transcription of *spalt* as well as *omb* (Figures 4, 6B, and 6C). All of these results suggest that the *spalt* and *omb* genes respond in distinct ways to different threshold concentrations of DPP and allow us to interpret the borders of *spalt* and *omb* expression as contour lines of a DPP gradient landscape.

The patterns of *omb* and *spalt* expression surrounding ectopic DPP-expressing cells also allow us to assess whether the signaling activity of DPP is modulated by other influences that act in a polarized or localized fashion within the wing primordium. As described above, it appears that DPP signaling is modulated in the dorsal embryonic ectoderm by a competing, opposing activity encoded by the gene *sog* expressed in neighboring, more ventral cells. However, we find that the halos of *spalt* and *omb* expression surrounding clones of ectopic DPP-expressing cells appear to be of constant width. Moreover, we have not observed an obvious difference in the size of these halos when ectopic DPP-expressing clones arise in different positions within the presumptive wing blade. Thus, all cells in the developing wing appear to be similarly responsive to DPP, suggesting that the graded distribution of secreted DPP protein may be the primary determinant of anteroposterior patterning in the wing.

It is important to note that our conclusions about distinct, direct outputs to DPP signaling in the wing are firm for *spalt* and *omb* expression, but may not apply uniformly to all other responses to the organizing activity of DPP. For example, both *omb* and *spalt* encode transcription factors (Pflugfelder et al., 1992; Kühnlein et al., 1994). Hence, in addition to controlling certain aspects of localized cytodifferentiation, such as the formation of wing vein primordia, they might also regulate the expression of other secreted signaling molecules that help elaborate the final cuticular pattern. Thus, our evidence that DPP acts as a gradient morphogen vis-à-vis *omb* and *spalt* does not exclude the possibility that other manifestations of its organizing activity may be mediated indirectly through the induction of downstream signals.

In conclusion, our findings suggest a model of wing development (Figure 7) in which a gradient of secreted DPP protein normally specifies at least three distinct states of genetic activity in wing cells: transcription of both *spalt* and *omb*, transcription of only *omb*, or transcription of neither gene. Wing cells are also subdivided into anterior and posterior compartments. Cells of the posterior compartment express the selector gene *engrailed* (*en*; Morata and Lawrence, 1975; Hama et al., 1990) and are thereby programmed to respond in different ways to DPP signaling (Zecca et al., 1995). Finally, EN activity in posterior cells also instructs them to express and secrete HH protein and hence to induce anterior cells at short range across the compartment boundary to express high levels of other proteins, particularly the transcription factor CI (Johnson et al., 1995). Thus, as diagrammed in Figure 7, the sequential activities of EN, HH, and DPP subdivide the developing wing into at

least seven distinct domains, each expressing a unique constellation of the transcription factors EN, CI, Spalt, and OMB, with much of this diversification resulting from the graded signaling activity of DPP.

Experimental Procedures

Receptor Transgenes

Wild-type *punt* and *tkv*. To generate the *UAS–punt* and the *UAS–tkv* transgenes, we cloned the SnaBI–HindIII fragment of cDNA *STK-C.7* (positions 387–2046; Ruberte et al., 1995) and a 2.5 kb EcoRI–DraI fragment of cDNA *STK-A* (Nellen et al., 1994) together with a DNA fragment containing the transcriptional termination sequences of the *Tubα1* gene (Struhl and Basler, 1993) into *pUAST* (Brand and Perrimon, 1993).

tor–punt and *tor–tkv*. The sequences of *UAS–punt* and *UAS–tkv* that encode the extracellular and transmembrane domains of Punt and TKV were replaced with the sequences encoding the extracellular and transmembrane domains of TOR^{4021} (Dickson et al., 1992). The fusion sites are immediately C-terminal to the transmembrane domains and have the expected sequences of RIRKQ for Punt and RILVRKQ for TKV, in which the first R represents R421 of TOR and the underlined residues are created by the linker.

tkv^{Q253D}. To mimic the activated phosphorylation state of the TGFβ type I receptor, we replaced all hydroxyamino acids serine and threonine of the TKV GS domain with the acidic amino acids aspartate and glutamate, respectively, in various combinations (D. N., unpublished data). None of these mutated forms of TKV displayed in vivo activity. A similar analysis has recently been reported for the TGFβ type I receptor (Wieser et al., 1995), indicating that analogous mutations of the GS domain of the TGFβ receptor impair phosphorylation activity. However, Wieser et al. found that replacement of the distal-most threonine residue (position 204) with aspartate resulted in an increased activity of the TGFβ type I receptor. Although this threonine residue is not conserved in most other type I receptors, we replaced the glutamine residue present at this position in TKV (Q253) by an aspartate. Surprisingly, this variant of TKV, designated TKV^{Q253D}, was constitutively active in all assays.

Ectopic Expression

Dorsalization of embryonic epidermis was as follows. Homozygous *69B* females (Brand and Perrimon, 1993) were crossed to males bearing a *UAS–dpp* or one or two receptor transgenes. Embryos were allowed to develop at 29°C, and their larval cuticles were mounted for compound microscopy. To test the activity of TOR–TKV and TOR–Punt in a *dpp* null mutant background, both transgenes were recombined onto a dpp^{H61} mutant chromosome and crossed to a dpp^{H61} stock homozygous for *69B*.

Labial induction. Transgene expression was induced using an *hsp70–GAL4* line, as in Ruberte et al. (1995).

Generation of FLP-out clones. A FLP-out cassette containing the rat *CD2* coding sequence and a y^+ minigene flanked by two FRT sites (Zecca et al., 1995) was inserted between the *UAS* promoter and the coding sequence. Transformants bearing such FLP-out transgenes were crossed to females of either the genotype *y w hsp70–flp omb–lacZ; +; C765* or the genotype *y w hsp70–flp; CyO spalt–lacZ; C765*. The resulting progeny were subjected to a mild heat shock (30 min at 34°C) during first, second, or third larval instar. Wing discs were removed during late third instar, fixed, and stained for CD2 and *lacZ* expression as described previously (Zecca et al., 1995). To induce clones expressing simultaneously TOR–Punt and TOR–TKV, larvae of the genotype *y w hsp70–flp omb–lacZ; UAS>CD2,y^+>tor–punt/UAS>tor–tkv; C765/+* were used. *C765*-driven expression of the *UAS* promoter results in significantly higher levels of expression in wing discs than those obtained from the *Tubα1* promoter (as in Zecca et al., 1995). This is reflected in the higher levels of CD2 protein produced from *UAS>CD2,y^+>* transgenes in a *C765* background compared with those produced from *Tubα1>CD2,y^+>* transgenes (data not shown).

Clones Lacking *tkv* Function

Marked clones of cells mutant for *tkv* were generated by FLP-mediated recombination (Xu and Rubin, 1993). Larvae of the genotype *y w hsp70–flp omb–lacZ/+; $tkv^{8\prime2}$ FRT40/πM FRT40* were subjected to a heat shock (30 min at 34°C) to induce mitotic recombination. Resulting late third instar larvae were subjected to a second, severe heat shock (1 hr at 37°C) to induce πM expression. After a recovery period of 1 hr, imaginal discs were fixed and double stained for πM and *lacZ* expression. Clones induced as late as 15 hr before dissection at end of third instar (latest timepoint tested) have lost *omb–lacZ* expression.

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References

Basler, K., and Struhl, G. (1994). Compartment boundaries and the control of *Drosophila* limb pattern by hedgehog protein. Nature *368*, 208–214.

Basler, K., Christen, B., and Hafen, E. (1991). Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing Drosophila eye. Cell *64*, 1069–1081.

Bienz, M. (1994). Homeotic genes and positional signaling in the *Drosophila* viscera. Trends Genet. *10*, 22–26.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., and Gelbart, W.M. (1991). An extensive 3′ cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGFβ family in *Drosophila*. Development *111*, 657–666.

Boveri, T. (1902). Ueber mehrpolige Mitosen als Mittel zur Analyse des Zellkerns. Verh. Phys. Med. Ges. Würzburg *35*, 67–90.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Brummel, T.J., Twombly, V., Marqués, G., Wrana, J.L., Newfeld, S.J., Attisano, L., Massagué, J., O'Connor, M.B., and Gelbart, W.M. (1994). Characterization and relationship of Dpp receptors encoded by the *saxophone* and *thick veins* genes in Drosophila. Cell *78*, 251–261.

Capdevila, J., and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. EMBO J. *13*, 4459–4468.

Dickson, B., Sprenger, F., and Hafen, E. (1992). Prepattern in the developing *Drosophila* eye revealed by an activated torso-sevenless chimeric receptor. Genes Dev. *6*, 2327–2339.

Di Fiore, P.P., Pierce, J.H., Kraus, M.H., Segatto, O., King, C.R., and Aaronson, S.A. (1987). erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science *237*, 178–182.

Fan, C.-M., and Tessier-Lavigne, M. (1994). Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a Hedgehog homolog. Cell *79*, 1175–1186.

Ferguson, E.L., and Anderson, K.V. (1992). decapentaplegic acts as a morphogen to organize dorsal–ventral pattern in the Drosophila embryo. Cell *71*, 451–461.

Francois, V., Solloway, M., O'Neill, J.W., Emery, J., and Bier, E. (1994). Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the short gastrulation gene. Genes Dev. *8*, 2602–2616.

Grimm, S., and Pflugfelder, G.O. (1996). Control of the gene optomotor-blind in *Drosophila* wing development by decapentaplegic and wingless. Science, *271*, 1601–1604.

Gurdon, J.B., Harger, P., Mitchell, A., and Lemaire, P. (1994). Activin

signalling and response to a morphogen gradient. Nature *371*, 487–492.

Hama, C., Ali, Z., and Kornberg, T.B. (1990). Region-specific recombination and expression are directed by portions of the *Drosophila* engrailed promoter. Genes Dev. *4*, 1079–1093.

Holley, S.A., Jackson, P.D., Sasai, Y., Lu, B., De Robertis, E.M., Hoffmann, F.M., and Ferguson, E.L. (1995). A conserved system for dorsal-ventral patterning in insects and vertebrates involving sog and chordin. Nature *376*, 249–253.

Hoppler, S., and Bienz, M. (1995). Two different thresholds of wingless signaling with distinct developmental consequences in the *Drosophila* midgut. EMBO J. *14*, 5016–5026.

Ingham, P.W. (1994). Pattern formation: hedgehog points the way. Curr. Biol. *4*, 374–350.

Ingham, P.W., and Fietz, M.J. (1995). Quantitative effects of hedgehog and decapentaplegic activity on the patterning of the *Drosophila* wing. Curr. Biol. *5*, 432–440.

Jiang, J., and Struhl, G. (1995). Protein kinase A and hedgehog signaling in Drosophila limb development. Cell *80*, 563–572.

Johnston, R.L., and Tabin, C. (1995). The long and short of *hedgehog* signaling. Cell *81*, 313–316.

Johnson, R.L., Grenier, J.K., and Scott, M.P. (1995). Patched overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. Development *121*, 4161–4170.

Kühnlein, R.P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J.F., Gehring, W.J., Jäckle, H., and Schuh, R. (1994). Spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. EMBO J. *13*, 168–179.

Lawrence, P.A. (1966). Gradients in the insect segment: the orientation of hairs in the milkweed bug *Oncopeltus fasciatus*. J. Exp. Biol. *44*, 607–620.

Lawrence, P.A. (1972). The development of spatial patterns in the integument of insects. In Developmental Systems: Insects, Volume 2, S.H. Counce and C.H. Waddington, eds. (London: Academic Press), pp. 157–209.

Lawrence, P.A., Crick, F.H.C., and Munro, M. (1972). A gradient of positional information in an insect, Rhodnius. J. Cell Sci. *11*, 815–853.

Letsou, A., Arora, K., Wrana, J.L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F.M., Gelbart, W.M., Massagué, J., and O'Connor, M.B. (1995). Drosophila Dpp signaling is mediated by the *punt* gene product: a dual ligand-binding type II receptor of the TGFβ receptor family. Cell *80*, 899–908.

Li, W., Ohlmeyer, J.T., Lane, M.E., and Kalderon, D. (1995). Function of protein kinase A in hedgehog signal transduction and Drosophila imaginal disc development. Cell *80*, 553–562.

Massagué, J., Attisano, L., and Wrana, J.L. (1994). The TGFβ family and its composite receptors. Trends Cell Biol. *4*, 172–178.

Masucci, J.D., Miltenberger, R.J., and Hoffmann, F.M. (1990). Pattern-specific expression of the *Drosophila* decapentaplegic gene in imaginal disks is regulated by 3′ cis-regulatory elements. Genes Dev. *4*, 2011–2023.

Morata, G., and Lawrence P.A. (1975). Control of compartment development by the engrailed gene in *Drosophila*. Nature *255*, 614–617.

Morgan, T.H. (1897). Regeneration in *Allolobophora foetida*. Roux's Arch. Dev. Biol. *5*, 570–586.

Nellen, D., Affolter, M., and Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by *decapentaplegic*. Cell *78*, 225–237.

Padgett, R.W., St Johnston, R.D., and Gelbart, W.M. (1987). A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature *325*, 81–84.

Pan, D., and Rubin, G.M. (1995). cAMP-dependent protein kinase and *hedgehog* act antagonistically in regulating *decapentaplegic* transcription in Drosophila imaginal discs. Cell *80*, 543–552.

Panganiban, G.E.F., Reuter, R., Scott, M.P., and Hoffmann, F.M. (1990). A *Drosophila* growth factor homolog, decapentaplegic, regulates homeotic gene expression within and across germ layers during midgut morphogenesis. Development *110*, 1041–1050.

Parkin, N.T., Kitajewski, J., and Varmus, H.E. (1993). Activity of Wnt-1 as a transmembrane protein. Genes Dev. *7*, 2181–2193.

Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J.L., Attisano, L., Szidonya, J., Cassill, J.A., Massagué, J., and Hoffmann, F.M. (1994). Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a *decapentaplegic* receptor. Cell *78*, 239–250.

Pflugfelder, G.O., Roth, H., and Poeck, B. (1992). A homology domain shared between *Drosophila* optomotor-blind and mouse Brachyury is involved in DNA binding. Biochem. Biophys. Res. Commun. *186*, 918–925.

Ruberte, E., Marty, T., Nellen, D., Affolter, M., and Basler, K. (1995). An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo. Cell *80*, 889–897.

Siegfried, E., and Perrimon, N. (1994). *Drosophila* wingless: a paradigm for the function and mechanism of Wnt signaling. Bioessays *16*, 395–404.

Smith, W.C., and Harland, R.M. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. Cell *67*, 753–765.

Spemann, H. (1938). Embryonic Development and Induction (New Haven, Connecticut: Yale University Press).

Staehling-Hampton, K., and Hoffmann, F. (1994). Ectopic decapentaplegic in the *Drosophila* midgut alters the expression of five homeotic genes, dpp, and wingless, causing specific morphological defects. Dev. Biol. *164*, 502–512.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E., and Bate, M. (1994a). Dpp induces mesodermal gene expression in *Drosophila*. Nature *372*, 783–786.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., and Hoffmann, F.M. (1994b). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by decapentaplegic but not 60A. Cell Growth Differ. *5*, 585–593.

St Johnston, R.D., and Gelbart, W.M. (1987). decapentaplegic transcripts are localized along the dorsal-ventral axis of the *Drosophila* embryo. EMBO J. *6*, 2785–2791.

St Johnston, D., and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell *68*, 201–219.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. Cell *72*, 527–540.

Stumpf, H. (1966). Mechanism by which cells estimate their location within the body. Nature *212*, 430–431.

Sun, Y.H., Tsai, C., Green, M.M., Chao, J., Yu, C., Jaw, T.J., Yeh, J., and Bolshakov, V.N. (1995). White as a reporter gene to detect transcriptional silencers specifying position-specific gene expression during *Drosophila melanogaster* eye development. Genetics *141*, 1075–1086.

Turing, A.M. (1952). The chemical basis of morphogenesis. Phil. Trans. Roy. Soc. (Lond.) *237*, 37–72.

Vincent, J.-P. (1994). Morphogens dropping like flies? Trends Genet. *10*, 383–385.

Vincent, J.-P., and Lawrence, P.A. (1994). Drosophila *wingless* sustains *engrailed* expression only in adjoining cells: evidence from mosaic embryos. Cell *77*, 909–915.

Wagner-Bernholz, J.T., Wilson, C., Gibson, G., Schuh, R., and Gehring, W.J. (1991). Identification of target genes of the homeotic gene Antennapedia by enhancer detection. Genes Dev. *5*, 2467–2480.

Wall, N.A., and Hogan, B.L.M. (1994). TGFβ-related genes in development. Curr. Opin. Genet. Dev. *4*, 517–522.

Wieser, R., Wrana, J.L., and Massagué, J. (1995). GS domain mutations that constitutively activate TβR-I, the downstream signaling component in the TGFβ receptor complex. EMBO J. *14*, 2199–2208.

Wolpert, L. (1989). Positional information revisited. Development (Suppl.), 3–12.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J. (1994). Mechanism of activation of the TGFβ receptor. Nature *370*, 341–347.

Xu, T., and Rubin, G.M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development *117*, 1223–1237.

Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. Development *121*, 2265–2278.